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PROTEOMIC INVESTIGATION OF MOLECULAR BASIS OF STRESS AND DISEASE RESISTANCE IN CATTLE

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SASKATCHEWAN AGRICULTURE, FOOD AND RURAL REVITALIZATION

"Proteomic Investigation of Molecular Basis of Stress and Disease Resistance in Cattle"

Project # 20020145

Final Report
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by

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Abstract: It is known that stress as a result of weaning (putting calves away from mothers) and transportation can lead to fatal bovine respiratory disease (BRD). It has been shown that stressors can alter the physiological levels of different proteins, metabolites and small peptides such as hormones, chemokines and cytokines. These alterations can send information to Central Nervous System to take necessary action which then sends messages to appropriate organs/tissues/cells to respond accordingly. These messages can either activate or suppress the immune system as needed and failure to compensate for this by the body can lead to serious health related problems. However how this is happening, what is the extent of different stressors to the susceptibility of a disease or how some individual can be resistant while others are susceptible to a particular disease though under the influence of similar level of same stressor. Finding answers to all these questions is very important a) to understand the mechanism of stress dependent disease susceptibility, b) to find ways to identify if an individual is susceptible to a disease or not, and c) to develop better intervention. Fatal BRD has often been associated with a primary viral infection which then compromises host immunity in ways that enhance the severity of a secondary bacterial infection. A primary viral infection such as bovine herpes virus-1 (BHV-1) followed by a secondary bacterial (e.g. *M. haemolytica*) recreates fatal BRD which was used as a model for the current program. It has been very difficult, however, to measure the impact of stress on BRD. Our main goal is to provide knowledge and develop strategies to identify the cause-effect relationship of stress dependent disease susceptibility in a model of BRD which can then be extended to study the correlation between stress and disease susceptibility in any other species. We identified the proteomic, metabonomic and elemental profiles in serum samples of BHV-1 infected and uninfected Abrupt Weaned (AW, stressed) and Pre-conditioned (PC, control) calves. Proteomic studies confirmed earlier observations and identified a group of acute phase proteins which could be linked to stress. Discriminant analysis of metabolite profiles showed distinct differential trends following weaning as well as clear shift in the trend of metabolite profiles following viral infection as well as indicated marked differences in metabolite profiles between calves which died or survived following a secondary bacterial infection. The current study also identified a group of metabolite biomarkers such as amino acids, LDL, glucose, lactate and elemental biomarkers such as phosphorous, iron, copper and molybdenum which were differentially associated with stress, infection and disease outcome. Overall this study indicated that (a) we can predict stress condition and disease outcome by identifying biomarkers (b) a group of indicators for any particular condition are more dependable than a single biomarker, (c) metabolites are more sensitive to predict disease outcome over other methodologies attempted.

Executive Summary:

Mortality and morbidity due to BRD continues to be a major health problem in fall-placed calves despite increased vaccination and the routine usage of prophylactic antibiotics upon arrival at the feedlot. It has been estimated that the average veterinary care cost in the feedlot is $15 Canadian/animal (2002) with the majority of this cost attributed to BRD treatment.

A common epidemiological feature of BRD in fall-placed calves is that 15-30% of calves are diagnosed with acute BRD and as many as 1-2% of calves develop fatal or chronic pneumonia. Furthermore, the majority of BRD cases develop within the first 30-60 days after calves enter the feedlot. Stress associated with weaning, transport, social mixing, and dietary change has been identified as a factor that contributes significantly to BRD disease susceptibility. It has been very difficult, however, to measure the impact of stress on BRD infections. Our main goal is to provide knowledge and develop strategies to identify the cause-

effect relationship of stress dependent disease susceptibility in a natural model of BRD. We planned to identify the host biomarkers for stressors which were responsible for pathogen challenged respiratory disease susceptibility as well as the markers for disease outcome. These biomarkers will help (a) understanding the molecular basis of stress dependent disease susceptibility, (b) developing future diagnostics for disease susceptible cattle, (c) finding therapeutic targets and (d) to understand the stress associated with animal management and disease susceptibility.

More research was needed to determine if the proteins already identified in serum samples by other researchers can be used as specific indicators of stress and påthogen infection. Studies are also needed to determine if the composition and quantity of metabolites and elements present in serum along with protein markers can be used to enhance the specificity with which stress responses or the infection status of animals can be predicted. We hypothesized that specific protein, metabolite and elemental biomarkers present in serum can be associated with stress responses and host responses to BHV-1 respiratory infection. We used an established BRD model in conjunction with important stressors (weaning and transportation) to analyze respiratory disease susceptibility and outcome in cattle. To test our hypothesis, proteomic, metabonomic and elemental analysis approaches were used to identify and characterize serum biomarkers in animals.

In this model, calves were aerosol challenged with bovine herpesvirus-1 (BHV-1) followed by *M. haemolytica*. This combined viral-bacterial infection is known to induce fatal pneumonia in 50-70% of calves. In order to verify dependence of BRD fatality on the stress level of the calves we used two groups pre-conditioned and abrupt weaned calves to determine if eliminating the stress of weaning and dietary change (pre-conditioning) had a significant impact on BRD infection.

Our clinical analyses revealed that weaning and transport had a significant impact on BRD infection with fatal pneumonia in 12% of pre-conditioned calves [control] and 88% of freshly weaned calves [stressed]. We used serum samples from blood of the animals for the convenience of handling and collection for screening/diagnostic purposes. Also blood being connective tissue should have representative markers for overall physiological and immunological changes.

We identified the proteomic, metabonomic and elemental profiles in serum samples of BHV-1 infected and uninfected stressed and control calves. Proteomic studies confirmed earlier observations and identified a group of acute phase proteins which could be linked to stress. Discriminant analysis of metabolite profiles showed distinct differential trends following weaning as well as clear shift in the trend of metabolite profiles following viral infection as well as indicated marked differences in metabolite profiles between calves which died or survived following a secondary bacterial infection. The current study also identified a group of metabolite biomarkers such as amino acids, LDL, glucose, lactate and elemental biomarkers such as phosphorous, iron, copper and molybdenum which were differentially associated with stress, infection and disease outcome. Overall this study indicated that (a) we could predict stress condition and disease outcome (dead or survived) by identifying biomarkers (b) a group of indicators for any particular condition are more dependable than a single biomarker, (c) metabolites are more sensitive to predict disease outcome over other methodologies attempted. The current project will also provide more insights to the cattle producers and farmers in understanding the effects of handling related stress and disease susceptibility.

Data generated also helped us in creating a new bioinformatic database on stress dependent disease susceptibility in cattle in relation with human genes and orthologs which might lead to

identifying novel intervention and therapeutics. As well, it could support screening of healthy animals or the prediction of disease susceptibility in cattle.

Technical report:

The project has the following milestones as proposed and detailed below:

Milestones	Expected Dates	Status
Animal trials, Sample collection and grouping (for artificial stress experiment using Dexamethasone with young calves)	March – June 2003	Finished
Standardization of methodologies	July-Sept 2003	Finished
Animal trials (Weaned and control young calves for respiratory disease challenge model with BHV-1 and M. *haemolytica*), Grouping and sample collection.	Oct 2003-Dec 2004	Finished
Separation of proteins by 2D-GE, Excise spots/collecting fractions/Digest	July 2004-June 2005	Finished
Mass Spectrometry-1 (for characterization and identification of proteins) for Dex studies	Jan-June 2005	Finished
Mass Spectrometry-1 (for characterization and identification of proteins) for Stress/Disease studies	Jan-Dec 2005	Finished
Mass spectrometry-2 (more detailed characterization) for Dex studies	Jan-July 2005	Finished
Mass spectrometry-2 (more detailed characterization) for Stress/Disease studies	Jan-Dec 2005	Finished
Protein I.D. + expression level, cataloguing (database) and bioinformatics	Jan 2004-Feb 2006	Finished
Weaning studies *New	Sept 2003- August 2006	Finished
Metabonomic Studies *New	July 2005- August 2006	Finished
Elemental Studies *New	July 2005- August 2006	Finished

*New: **These studies were not part of the original plans but initiated in addition to what planned earlier to obtain more information regarding the stress and disease outcome at levels bevond protein.**

The current project addressed two important Goals: (a) to understand the potential of the omics methodologies and succesful establishment of these methodologies at VIDO and (b) to identify various biomarkers in serum for weaning stress and disease outcome.

Goal 1: Methodologies Used:

A) **Experiments planed to establish proteomic methodologies:** Immunosuprresion causes susceptibility to various infectious diseases following acute and chronic stressors. We designed an experiment with Dexamethasone (DEX, a synthetic adrenocortical steroid) which acts as immuno suppressant to mimic the effect of acute stress by injecting 4-mo old calves one time with Dex. In this study we injected one time the animals (n=3) with Dexamethasone at a dosage of 50 μg/kg and followed the time course of serum protein expression over next consecutive 3-

days. Comparative two dimensional gel electrophoresis (2D-GE) of serum proteins from DEX treated and un-treated animals from Days 0, 1, 2 and 3 suggest differential expression of various proteins. The kinetics and the mass spectrometric characterization of the protein expression were analyzed. While we succesfully established the proteomic methodologies no significant and biologically important results can be obtained from this study because of using small number of animals (n=3).

B) **Proteomic methodology:** Although 2-Dimensional Gel Electrophoresis (2D-GE) analysis has been successfully utilized for almost 30- years in protein profiling, it provides little information concerning protein identification. Recent advances in mass spectrometry (MS) and the establishment of genomic and protein databases have substantially increased the ease and speed with which proteins can be identified. The union of these technologies is the foundation for modern proteomic studies. A review of the different MS techniques and the role of MS in the Age of the Proteome were recently presented. .

We used 2D-GE in conjunction with MALDI and Q-TOF-MS to investigate differences in protein expression in serum samples of different animals from various groups proposed in the current project. The protein bands differentially expressed among various groups under investigation were selected, excised and in-gel digested. First round of protein identification was performed using a MALDI-TOF-MS peptide mass mapping approach. Proteins spots were excised, digested in-gel using a protease (trypsin), and the resultant peptide masses were measured. The observed peptide masses were searched against protein databases (NCBI, SWISS-PROT and TrEMBL), when no convincing hits are found data were searched against dbEST and the retrieved ESTs were queried in similarity searches of NCBI nr using BLAST.

We use web based MASCOT for the searches. Increased peptide mass accuracy has increased the success and selectivity of such searches. If the database query was unsuccessful, the protein was sequenced using tandem mass spectrometry (LC-MS/MS). During the LC-MS/MS experiment, only the peptide mass of interest is isolated or transmitted, thus discriminating against all other components of the mixture with different mass values. After isolation, the peptide was fragmented using a uni- or bi-molecular (collision gas) strategy. Fragments observed in the isolated peptide can then be rationalized to a sequence or used to search the databases using a sequence tag approach. LC/MS/MS and MALDI/MS/MS were used as needed for more difficult identifications, sequencing, and post translational modification determination. All mass spectrometric analyses were done at NRC-Plant Biotechnology Institute on campus (PBI).

C) **Metabonomic methodology:** Metabonomic methodology is the study of functional metabolites using Nuclear Magnetic Resonance technique. 1D- ^{1}H NMR spectral signals of the serum samples from abrupt weaned and pre-conditioned calves were recorded at a resonance frequency of 600.17 MHz on a Bruker Avance-600 spectrometer (Rheinstetten, Germany) at Saskatchewan Structural Science Centre (SSSC) on campus. The spectral profile was similar to that of published ^{1}H-NMR profile obtained from human, mouse and rat serum samples and the peaks were assigned to specific metabolites by comparing published data on human and mouse serum.

D) **Discriminant analysis**

Discriminant analysis was performed on the Fourier transformed 1-dimensional ^{1}H-NMR data sets for peak intensities of various identified metabolites. Two dimensional Eigen value-scores were constructed using StatistiXL package in order to establish the presence of any

patterns or clusters in the data as a result of the stress condition or disease outcome using serum samples collected before or after BHV-1 infection.

E) **Elemental Analysis**

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was used to quantitatively analyze the elements present in serum samples collected from the abrupt weaned and pre-conditioned-calves. We quantitatively determined the amount of 15-elements, including Li, B, Mg, P, Ca, Ti, Cr, Fe, Cu, Zn, Se, Sr, Mo, Cd, and Ba.

F) **Statistical analyses**

For serum protein, metabolite and element abundance data, 2-way ANOVA was employed after appropriate data transformation. All individual group comparisons were made using Tukey's method. The criterion for significance was $p < 0.05$ for all studies.

Goal 2: <u>Biomarkers Discovery:</u>

A) **Experiment 1.** Our next goal was to find the proteomic, metabonomic and elemental basis of weaning stress and BRD susceptibility. A group of young calves (n =30) has been separated into abrupt weaned (n=15) and pre-conditioned groups (n=15). Pre-conditioned or PC groups consisted of suckling calves which were removed from their mothers (weaned) two weeks prior to disease challenge. This two week interval was to reduce or eliminate potential psychological and physiological effects associated with breaking the maternal/nutritional bond. Abrupt Weaned or AW group consisted of suckling calves which were separated from their mothers one day prior (Day -1) to BHV-1 challenge (Day 0). On the same day (Day-1), calves in both groups were transported for a period of 3½ h to arrive at VIDO. Blood samples from AW and PC calves were collected on Day 0 (the day following weaning and prior to infection with BHV-1) and on days 1, 2 and 4 following BHV-1 infection. On Day 4 (when the susceptibility to fatal secondary bacterial infection is greatest) calves were challenged with *M. haemolytica*. A veterinarian conducted clinical evaluation (body temperature and weight) of all calves daily throughout the trial. Following bacterial challenge a subset of calves developed fatal pneumonia and calves were grouped based on disease outcome regardless of treatment prior to infection as either Dead or Survived. We selected this combined viral-bacterial challenge protocol as it is representative of infections found in natural conditions. Other samples such as Peripheral Blood Mononuclear Cells, nasal secretions, and lung lavage cells were also collected and used for other studies. Both serum haptoglobin and cortisol concentrations have been measured in the serum of weaned and control animals before and after viral and bacterial challenges. Haptoglobin is used as an indicator of disease susceptibility and cortisol as a measure of stress levels.

Though in general weaned animals are more susceptible but we did not observe any mortality among the calves in the study. Proteomic studies and other clinical scores also failed to show any significant difference between these two groups. We, therefore, designed a repeat experiment with 20-animals (n=10/group) and results showed 80% mortality in weaned animals compared to 40% in non-weaned animals. We also observed cortisolemia in both groups which may be because of transportation and cortisolemia sustained for pre-conditioned animals for 2-days. Serum from AW- and PC-calves were analyzed using proteomic, metabonomic and elemental analyses.

B) **Experiment 2.** We conducted a second experiment to investigate the stress of weaning specific to disruption of the maternal/nutritional bond, with no associated effect due to transport, environmental change, or social mixing/re-organization. Three groups were used (n=10/group):

never weaned (cow/calf remained together throughout the study period); pre-conditioned (weaned and conditioned for two weeks); Abrupt weaned. Cow/calf pairs were vaccinated (8-way Clostridial; MLV BVD/PI3/BHV-1) 2 wks prior to initiating the study to minimize potential effects of respiratory viral infections during the weaning period. All cow/calf pairs were housed in a single pen throughout the study; weaning was performed by removing selected cows from the pen. Serum samples were collected on experimental days 0 (day calves were abruptly weaned) and 1, 2 and 3. The results showed no significant difference in total cortisol among the groups. Serum samples from these animals were analyzed further to identify stress related proteins. We identified few proteins which could be associated specifically to abrupt weaning. Combined with observations from the first study (BRD trial), transportation is implicated as a major factor for inducing cortisolemia in calves.

EXPERIMENTAL PLANS:

1) Dexamethasone Trial:

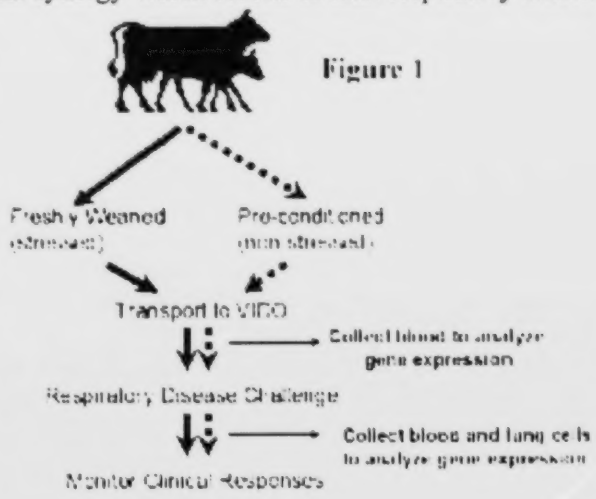
The experimental plan was to obtain six healthy young calves and administer DEX by injection to induce chemical stress as follows:

Table 1: Experimental Time Course

Day 0 (Control)	Day 1	Day 2	Day 3
-Collect serum and nasal secretion samples - Inject 50µg/kg of Dex/animal	-Collect serum and nasal secretion samples	-Collect serum and nasal secretion samples	-Collect serum and nasal secretion samples

2) Respiratory disease challenge of abrupt weaned and pre-conditioned cattle:

a) **Trial 1:** Disease models have been developed at VIDO to study the molecular mechanisms underlying the viral-bacterial synergy which results in fatal respiratory infections . To recreate the BRD complex commonly referred to as "shipping fever" we used two groups of calves. One group of suckling beef calves (n =15) was adapted for two weeks to the dietary change and social re-organization that occurs following weaning (pre-conditioned, **PC**). A second group of suckling beef calves (n = 15) was removed from their mothers 24 h prior to viral infection (abrupt weaned, **AW**) (Figure 1). Each group was sampled for serum, nasal secretions, and/or lung lavage cells (Table 2). Different samples were collected for doing various other experiments to understand the overall disease process, however for our current project we will


Pre-conditioned
Transport to VIDO
Collect blood to analyze gene expression
Respiratory Disease Challenge
Collect blood and lung cells to analyze gene expression
Monitor Clinical Responses
Figure 1

only concentrate on serum samples and later also Alveolar macrophages. The results did not show much severity in the disease outcome. There was also no mortality observed in either group. We therefore, repeat the experiments last fall as described in next section. For proteomics studies we use serum samples and observed some differences, however we could not use nasal secretion as they were contaminated with blood from damaged tissue in the nose.

Table 2: Experimental plan for respiratory challenge (Trial 1)

			Experimental Protocol				
Group	n	Treatment	Day 0 (Ctrl)	Day 1	Day 4	Day 5	Day 7
I	15	PC	Blood, nasal, lung lavage collected	BHV-1 aerosol challenge	Blood, nasal, lung lavage collected	M. *Haemolytica* aerosol challenge	Blood, nasal secretions collected
II	15	AW					

b) **Trial 2:** The repeat study was done as shown in Figure 1 with 10-animals per group. We collected serum samples from the animals as described in Table 3.

Table 3: Experimental plan for Second respiratory challenge (Trial 2)

			Experimental Protocol			
Group	n	Treatment	Day 0 (Ctrl)	Day 1	Days 2-4	Days 5-7
I	10	PC	Blood collected	Blood collected followed by BHV-1 aerosol challenge	Blood collected & aerosol challenge with M. *Haemolytica* on Day 4	Clinical monitoring and Blood collected
II	10	AW				

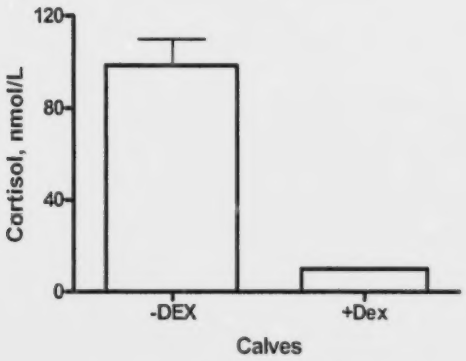
Repeat experiment was done with 10-animals per group. Groups are defined as mentioned for the first viral-bacterial synergy. Blood was collected once the animals were brought to VIDO on Day 0. Blood was also collected on Day 1 followed by BHV-1 challenge and also on days 2 to 4. On day 4 calves were challenged with bacteria (*M. haemolytica*) and blood was continued to be collected for days 5-7. Calves were clinically monitored after the bacterial challenge for next 7-days and a veterinarian scored for the lung pathogenecity of the dead animal. Body weight and temperatures were also measured for all calves for all the days.

RESULTS

Dexamethasone Trial

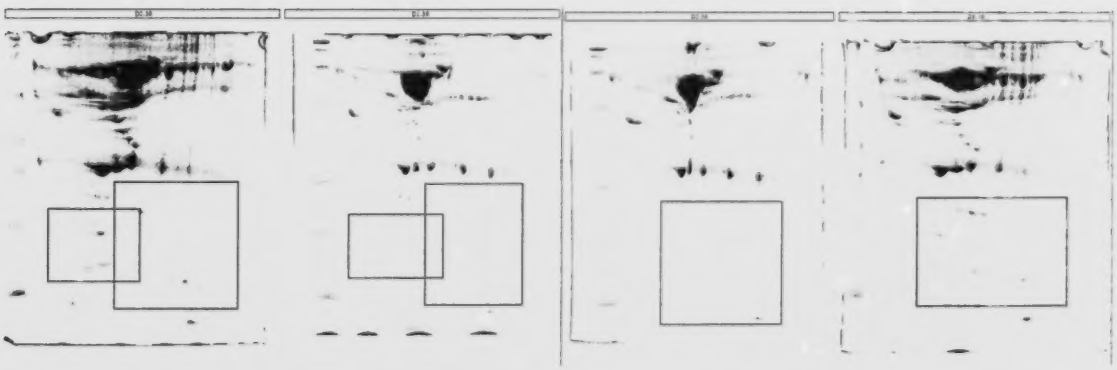
a) All serum samples from this trial were measured for cortisol concentration. It was observed that serum cortisol levels were higher in the controls than in the DEX treated animals.

Figure 2: Serum Cortisol Levels in Young Calves



b) 2D-GE studies revealed approximately 80 differentially expressed protein bands from the serum samples. Figure 3 is representative gel images and Figure 4 is a subset of those protein markers that were identified. The red boxed areas in Figure 3-indicates the regions of differentially expressed proteins and the proteins which were present on day 0 were repressed on days 1 and 2. Higher resolution gels for better identification are currently being done.

Figure 3: 2D Gel images of serum samples from control (Day 0) and Dex Treated (Days 1, 2 & 3) calves



Day 0 **Day 1** **Day 2** **Day 3**

c) All distinctly separated protein bands were analyzed using MALDI-TOF mass spectrometry. Mass spec data was searched in a protein database (SWISSPROT) and many bands were annotated. However, a portion of the proteins could not be identified. These were further analyzed using quadrupole time-of-flight (Q-TOF) mass spec. This data yielded both further characterization of unknown proteins and confirmation of identified proteins. Figure 4 below shows some of the important and already characterized proteins. They can act as biomarkers for DEX mimicked stress situation in cattle. More proteins are being analyzed and characterized.

Figure 4

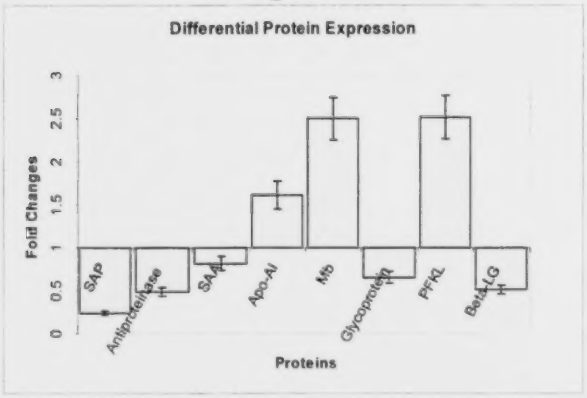
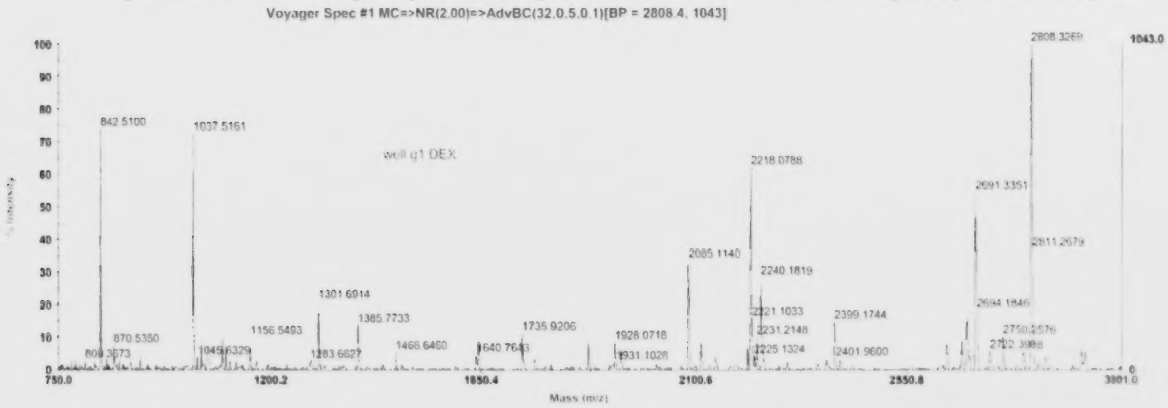


Figure 5: MALDI-TOF protein spectrum data identified as Apolipoprotein AI. This is a representative of the quality of mass spectra we use for characterizing the protein samples.



Respiratory Disease Challenge

a) **Trial 1**.

i) Clinical observations show that stressed calves lost more body weight than non-stressed calves (Figure 6).

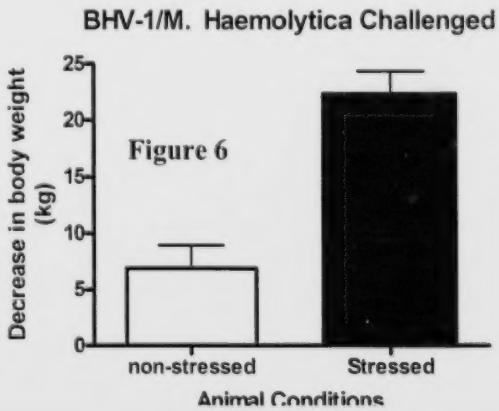
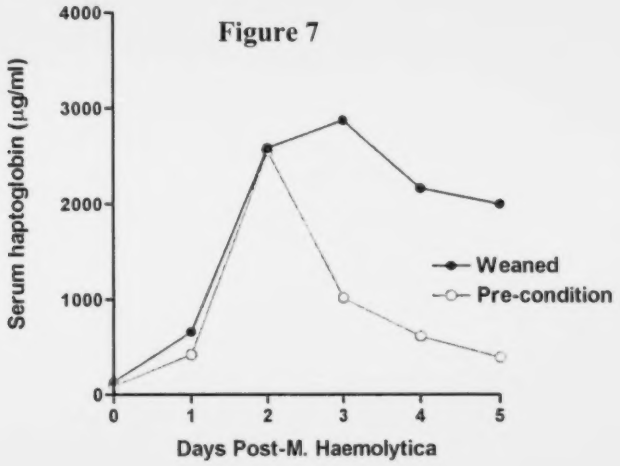


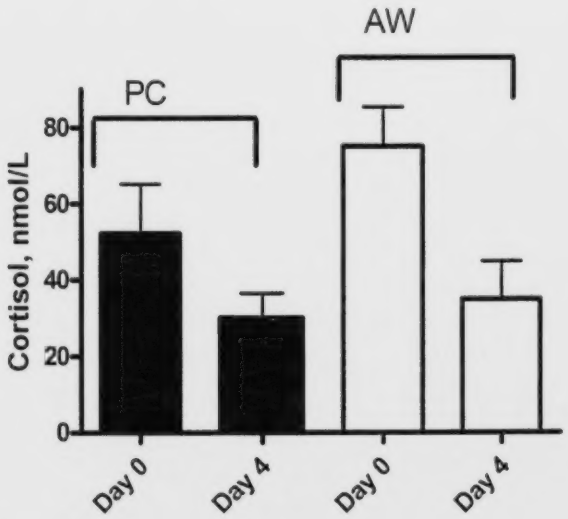
Figure 6

ii) Stressed calves also exhibited higher inflammation measured by serum haptoglobin levels (Figure 7).



iii) Serum cortisol levels were observed to be higher in stressed versus non-stressed animals. The levels did, however, decrease after challenge with BHV-1 in both stressed and non-stressed groups (Figure 8).

Figure 8. Cortisol level in serum samples on Days 0 ad 4 for PC and weaned group of animals from Trial 1



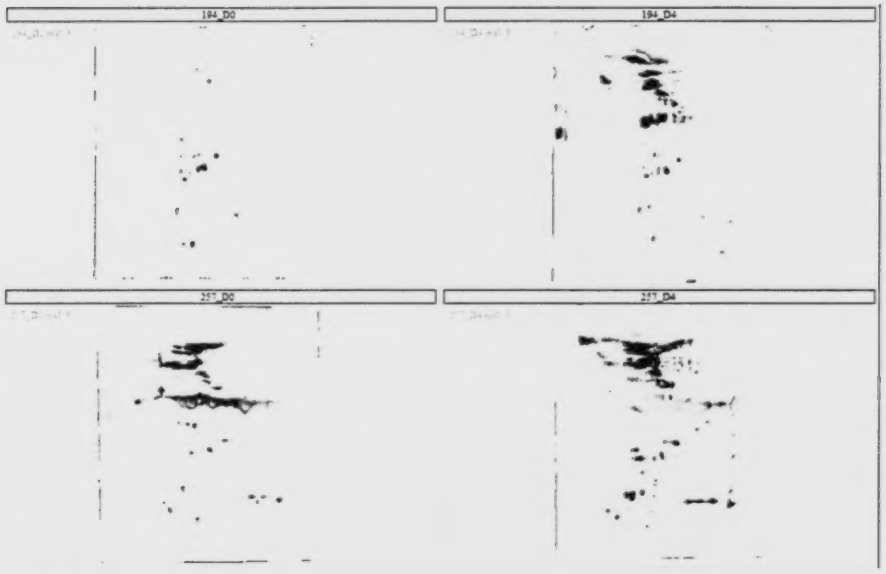
Serum cortisol level on day 0 for AW was higher than PC but not statistically significant ($p = 0.2$) however the data when analysed using one way analysis of variance showed that the means of experimental data for each group for days were significant ($p = 0.02$).

iv) The collected serum samples were then used for proteomic studies. A representative of the 2D-GE gels are shown in Figure 8. The animals were selected from a pre-exposed

low titre of BHV-1 and *M. haemolytica*. Protein samples from each group and treatment went through a standard proteomic experimental protocol as described below.

v) Gels were compared among various conditions and differential protein markers were identified,expression levels are evaluated and the protein bands are being characterized. Representative gels are shown below (Figure 9).

Figure 9: 2D-GE pattern of protein expression of post BHV-1 infection for (a) stressed and (b) non-stressed animal



vi) **Trial 2:** Despite all the clinical scores we did not observe any disease outcome in terms of mortality in the first trial. So the severity of challenge and the effectiveness of the model was not unequivocally established. Moreover, serum samples collected on day 0 before BHV-1 and Day-4 after BHV-1 infection could not resolve the issue if cortisolemia was sustained or not. We therefore collected blood to verify this on days 0 to 4. We also observed mortality following bacterial challenge. Increased mortality associated with abrupt weaning was characterized by a decrease in both survival time (Figure 10) and lung pathology.

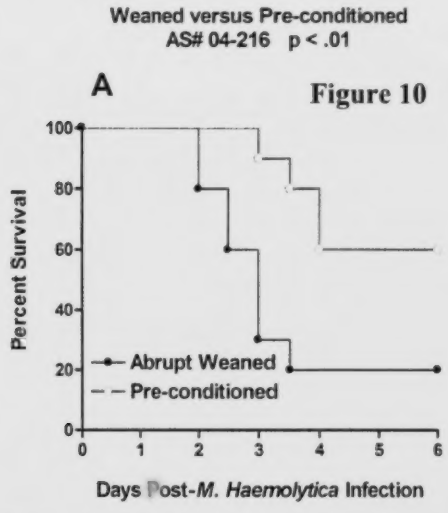
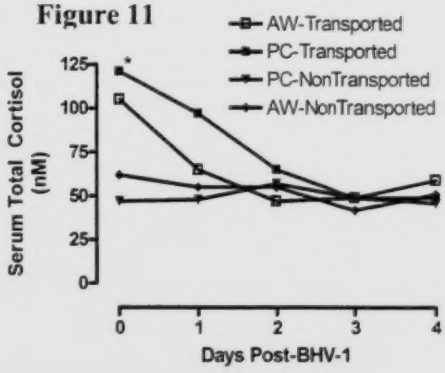
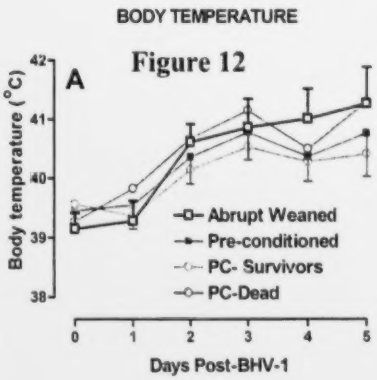


Figure 11



These observations suggested that endotoxemia and septic shock may contribute to bacterial-induced death at the rate of 40% for pre-conditioned and 80% for AW (Figure 10). The transportation of calves induced a sustained cortisolemia in the pre-conditioned calves but not the abrupt weaned calves (Figure 11). Thus, an increase in fatal bacterial respiratory infection was associated with an accelerated decline in serum cortisol levels. Cortisol is a potent regulator of pro-inflammatory cytokine transcription by acting as a negative regulator of NFκB activation. Therefore, we hypothesized that increased respiratory disease susceptibility is associated with enhanced pro-inflammatory responses in abrupt weaned calves. This hypothesis was supported by the microarray results performed for another studies and by the observation that body temperature (Figure 12) and IFN-γ levels in nasal secretions were significantly elevated during BHV-1 infection of abrupt weaned calves. A similar observation supported the role of IFN-γ in BRD. These innate immune responses were significantly increased despite no significant difference in virus shedding. Furthermore, increased IL-10 expression during BHV-1 infection of pre-conditioned calves is consistent with a reduction in pro-inflammatory responses. Microarray analysis revealed both conservation and differences in leukocyte gene expression following BHV-1 infection when abrupt weaned and pre-conditioned calves were compared. Conserved responses included an enhanced potential to respond to pathogen-associated molecular patterns through increased expression of toll-like receptors (TLRs). TLR induction of innate immune responses is mediated primarily through activation of NFkB. We also observed differential expression of β-defensin 5, a host defense peptide which is induced by TLR4 signaling, in abrupt weaned calves and is consistent with reduced cortisol levels. In conclusion, the stress of abrupt weaning significantly enhances the viral-bacterial synergy that leads to fatal bacterial respiratory infection. Microarray analysis and clinical data support the conclusion that differential regulation of pro-inflammatory responses is a major mechanism contributing to this increased disease susceptibility. Comparative proteomic studies with serum samples from these animals suggest some unique protein expression patterns following BHV-1 infection (Figure 13).



vii) While protein data before bacterial challenge was not significant enough to predict the outcome of infection for calves which died or survived metabonomic data was very successful in discriminating the metabolic patterns for calves which survived or died. Figure 14 shows discriminant analyses which shows clear differences in metabolic profile between various stress groups or calves which were grouped based on mortality.

Figure 13: 2D Gel Images

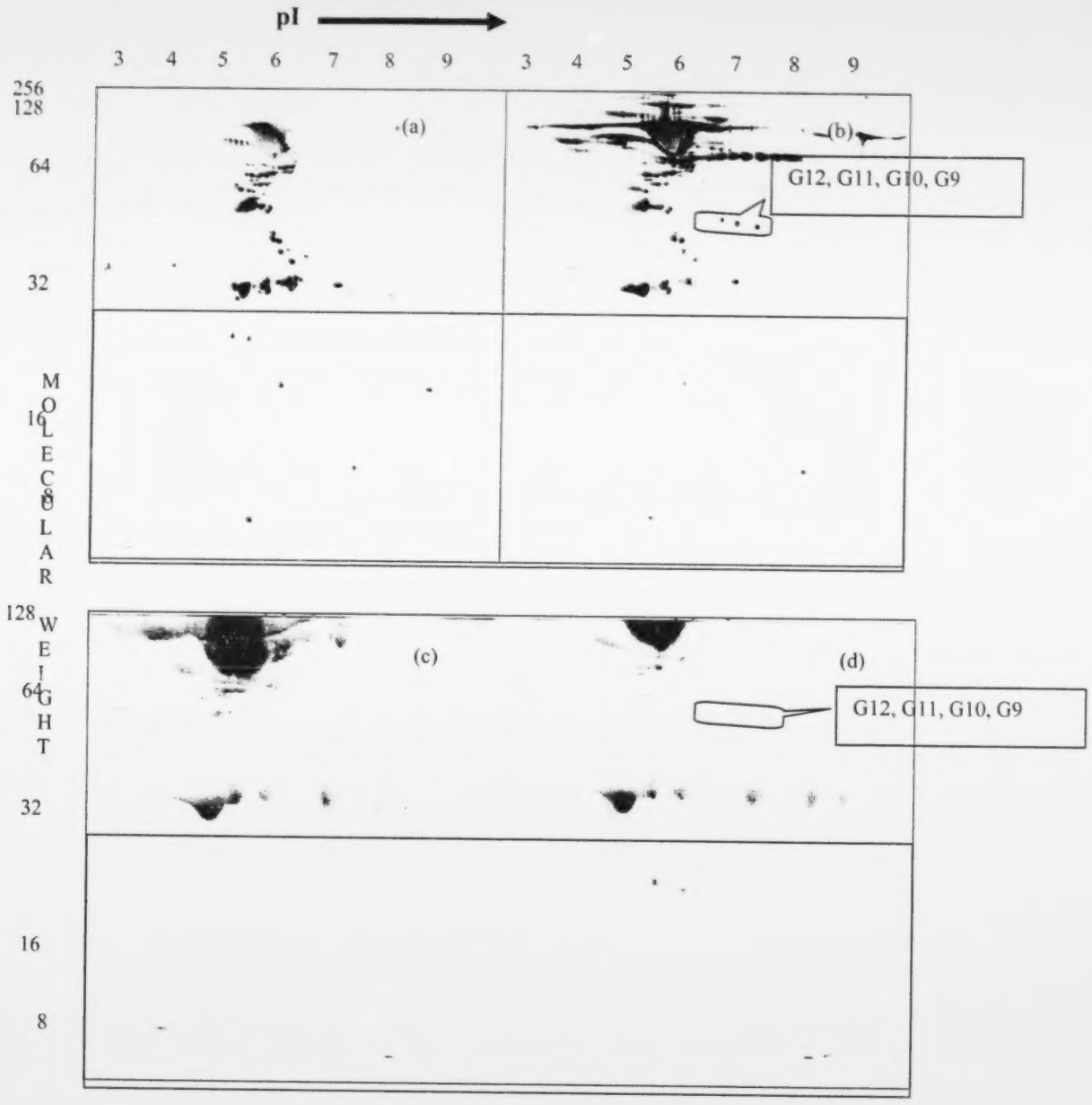
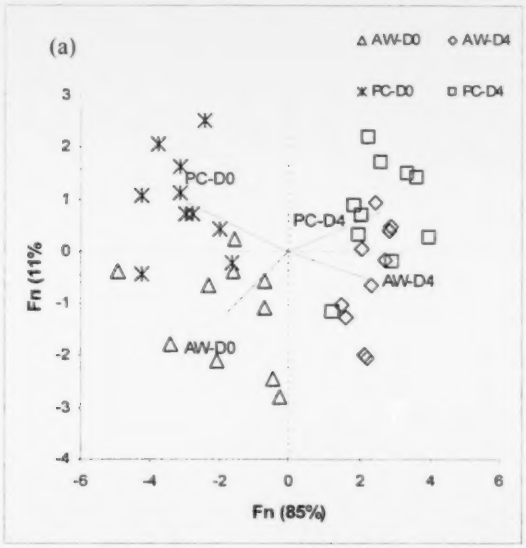


Figure 14. Discriminant Analyses comparing (a) AW- and PC-calves before (Day 0) and after (Day 4) BHV-1 infection and (b) calves which survived (S) and Died (D) using metabolite abundance in serum on Day 0 and Day 4.



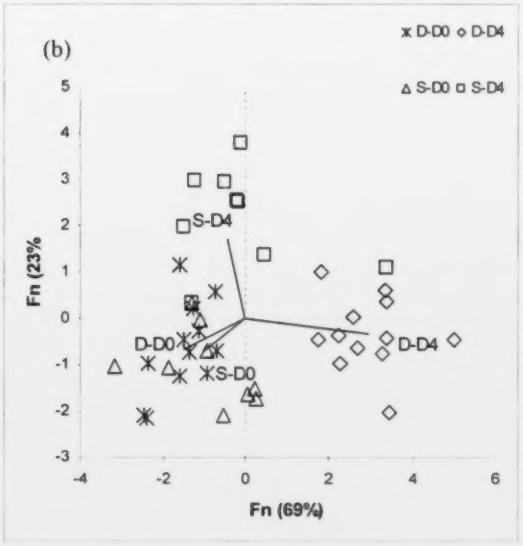


Table 4 shows the mass spectrometric characterization of the significant ($p \leq 0.05$) proteins

Table 4. Proteins characterized from the Bos Taurus serum proteome

Protein Name	SWISS-PROT or NCBInr accession number	Experimental pI/MW	Theoretical pI/MW	Sequence

Apolipoprotein C-III	S13188	5.14/6762	4.7/7548	(K)DYWSSFK(G) (R)DWMTESFSSLK(D) (R)DWMTESFSSLK(D) (K)DALSSVQESQVAQQAR(D)
Apolipoprotein C-III	S13188	5.0/7543	4.7/7548	(K)DYWSSFK(G) (R)DWMTESFSSLK(D) (R)DWMTESFSSLK(D) (K)DALSSVQESQVAQQAR(D)
Serum amyloid A protein (SAA)	_P35541	6.59/11092	6.9/12603	(R)FTDPLFK(G) (R)GNYDAAQR(G) (K)GADKYFHAR(G) (R)ADQAANEWGR(S) (K)GTTSGQGQEDSR(A) (K)DPNHFRPAGLPDKY(-)
Similar to Haptoglobin precursor	XP_587897	7.36/46442	7.83/45643	(R)IIGGSLDAK(G) (K)GSFPWQAK(M) (K)MVSQHNLISGATLINER(W) (K)MVSQHNLISGATLINER(W) Oxidation (M) (R)WLLTTAK(N) (K)NLYLGHSSDKK(A) (K)DITPTLR(L) (K)NQLVEVEK(V) (R)QKVPVNDK(V) (K)VMPICLPSK(D) Oxidation (M) (R)VGYVSGWGR(N) (K)YVMLPVADQDK(C) (K)YVMLPVADQDK(C) Oxidation (M) (K)SCAVAEYGVYVK(V)
Similar to Haptoglobin precursor	XP_587897	7.17/46529	7.83/45643	(K)HPVDQVQR(I) (K)GSFPWQAK(M) (K)MVSQHNLISGATLINER(W) (K)MVSQHNLISGATLINER(W) Oxidation (M) (K)NLYLGHSSDK(K) (K)NLYLGHSSDKK(A) (K)AKDITPTLR(L) (K)NQLVEVEK(V) (K)VVLHPDHSK(V) (K)VMPICLPSK(D) (R)VGYVSGWGR(N) (K)YVMLPVADQDK(C) (K)YVMLPVADQDK(C) Oxidation (M) (K)SCAVAEYGVYVK(V)
Similar to	XP_587897	6.97/46835	7.83/45643	(K)HPVDQVQR(I)

Haptoglobin precursor				(K)GSFPWQAK(M) (K)MVSQHNLISGATLINER(W) (K)MVSQHNLISGATLINER(W) Oxidation (M) (K)NLYLGHSSDK(K) (K)NLYLGHSSDKK(A) (K)AKDITPTLR(L) (K)NQLVEVEK(V) (K)VVLHPDHSK(V) (K)VMPICLPSK(D) (K)VMPICLPSK(D) Oxidation (M) (R)VGYVSGWGR(N) (K)YVMLPVADQDK(C) (K)YVMLPVADQDK(C) Oxidation (M) (K)YQDDTCYGDAGSAFVVHDK(E) (K)SCAVAEYGVYVK(V)
Similar to Haptoglobin precursor	XP_587897	6.76/47363	7.83/45643	(K)HPVDQVQR(I) (K)GSFPWQAK(M) (K)MVSQHNLISGATLINER(W) (K)MVSQHNLISGATLINER(W) Oxidation (M) (K)NLYLGHSSDK(K) (K)NLYLGHSSDKK(A) (K)AKDITPTLR(L) (K)NQLVEVEK(V) (K)VVLHPDHSK(V) (K)VMPICLPSK(D) (K)VMPICLPSK(D) Oxidation (M) (R)VGYVSGWGR(N) (K)YVMLPVADQDK(C) (K)YVMLPVADQDK(C) Oxidation (M) (K)YQDDTCYGDAGSAFVVHDK(E) (K)SCAVAEYGVYVK(V)
Hemoglobin alpha chain	CAB56828	7.5/9886	8.2/ 15157	(R)MFLSFPTTK(T) (R)MFLSFPTTK(T) Oxidation (M) (K)LRVDPVNFK(L) (K)VGGHAAEYGAEALER(M) (K)TYFPHFDLSHGSAQVK(G) (K)AVEHLDDLPGALSELSDLHAHK(L)
Similar to Haptoglobin precursor	XP_587897	5.95/24615	7.83/45643	(K)APEIANSHVEYSVR(Y) (K)LHAGNGVYTFNNK(Q) (K)IENGYVEYLVR(Y) (R)YQCKPYYTLR(T) (R)TCGDGVYTFNSK(K)

				(K)LPECEAVCGKPK(H) (K)HPVDQVQR(I)
Apolipoprotein A-I	AAB21444	6.53/24415	7.83/45643	(K)ETASLR(Q) (R)AHVETLR(Q) (K)VAPLGEEFR(E) (K)LSPLAQELR(D) (K)AKPVLEDLR(Q) (R)QGLLPVLESLK(V) (K)EGGGSLAEYHAK(A) (K)DFATVYVEAIK(D) (K)WHEEVEIYR(Q) (K)VQPYLDEFQK(K) (R)QQLAPYSDDLR(Q) (K)KWHEEVEIYR(Q) (R)DYVAQFEASALGK(Q) (R)VKDFATVYVEAIK(D) (K)LLDNWDTLASTLSK(V) (R)EQLGPVTQEFWDNLEK(E) (K)VREQLGPVTQEFWDNLEK(E)
Hemoglobin alpha chain	CAB56828	8.67/8710	8.2/ 15157	(R)MFLSFPTTK(T) Oxidation (M) (K)LRVDPVNFK(L) (K)VGGHAAEYGAEALER(M) (K)TYFPHFDLSHGSAQVK(G) (K)AVEHLDDLPGALSELSDLHAHK(L)

(b) Weaning in the absence of Transportation: Though the current model clearly established that weaning stress induces disease susceptibility in young calves and identified some genetic markers, it was not possible to quantify the exact effects of each stressor. The current model of weaning and pre-conditioned groups experienced transportation and new environment related stress on top of their weaning, social re-organization and nutritional/dietary changes.

There are reports which suggested that transport can be an important stressor to set the stage for BRD and can have impact on blood composition. Therefore, we designed a second experiment where we eliminated transportation of animal and as a result stressor related to new environment was removed too. This experiment was designed in collaboration with Dr. Joseph Stookey of Western College of Veterinary Medicine. It was documented that abrupt weaning (separation of suckling calves from their dams) causes a prolonged psychological stress response. This stress response manifests as an increase in vocalization by both calves and cows and a significant increase in time spent walking by the calf. Consequently, time spent eating and resting also decreases for abruptly weaned calves. Statistically, these changes in behavior return to baseline values after 4 to 5 days of weaning.

We performed the study with 30-cow/calf pairs at the university owned Goodale farm and vaccinated (8-way Clostridial; MLV BVD/PI3/BHV-1) two weeks prior to initiating the study to minimize potential effects of respiratory viral infections during the weaning period. All cow/calf pairs were housed in a single pen throughout the study and separation of cow and calf (weaning) was performed by removing selected cows from the pen. Thus, the stress of weaning was specific to disruption of the maternal/nutritional bond with no associated effect due to

transport, environmental change, or social mixing/re-organization. Assignment of calves to groups was by formal randomization (random number table) and the laboratory remained blind to the experimental groups until sample analysis was complete. There were 3-groups (n=10/group): **Group I - Never Weaned (NW):** Calves remained with their mothers throughout the study. These calves provided controls for possible effects due to handling during sample collection and also provided a comparison with suckling calves. **Group II - Pre-conditioned (PC):** Cows were removed from calves (traditionally weaned) two weeks prior to the start of the trial. This two week period was selected as an appropriate interval to eliminate potential psychological and physiological effects associated with breaking the maternal/nutritional bond. **Group III - Abrupt Weaned (AW):** Cows were removed from the calves at the beginning of the experimental sample collection (Experimental day 14). This group should display the full range of behavioral responses associated with breaking the maternal/nutritional bond and the most pronounced physiological changes if these occur during weaning.

The results showed that there was no significant change in total cortisol among these groups. These results, when compared with our other trial of BRD, clearly indicate that transportation can be a major stressor for inducing cortisolemia in calves and as suggested earlier that cortisol can be used as an indicator of stress . Serum samples from these animals were analyzed further to identify any stress related protein.

Figure 15. 2D-GE for AW, NW and PC groups

Figure 16. Zoomed in segments of Figure 14.

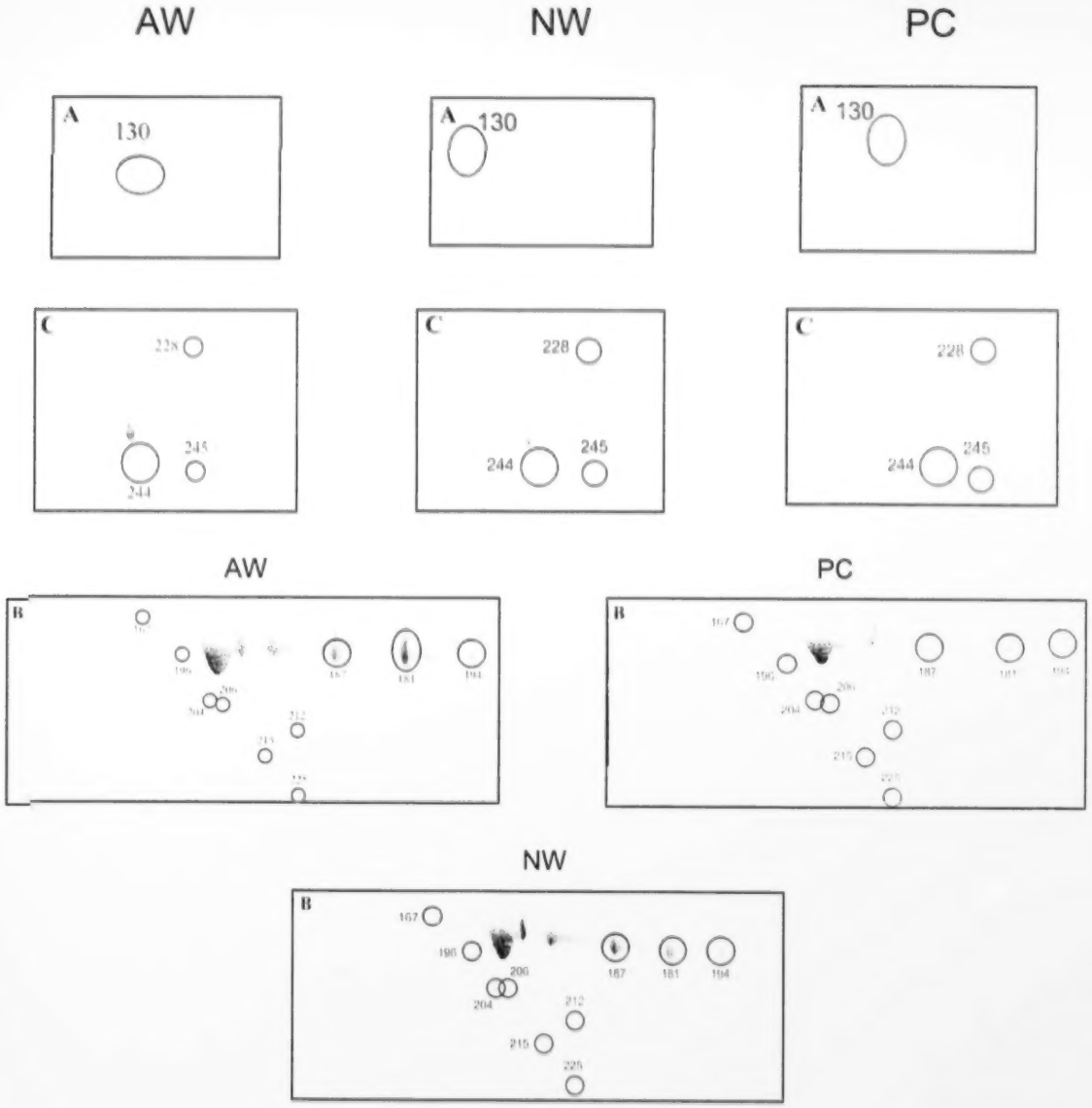


Figure 17. Kinetics of few representative protein bands are shown for three groups. Spot identifications which were characterized by mass spectrometry are shown besides the spot. Spots without identifications are unknown at this stage.

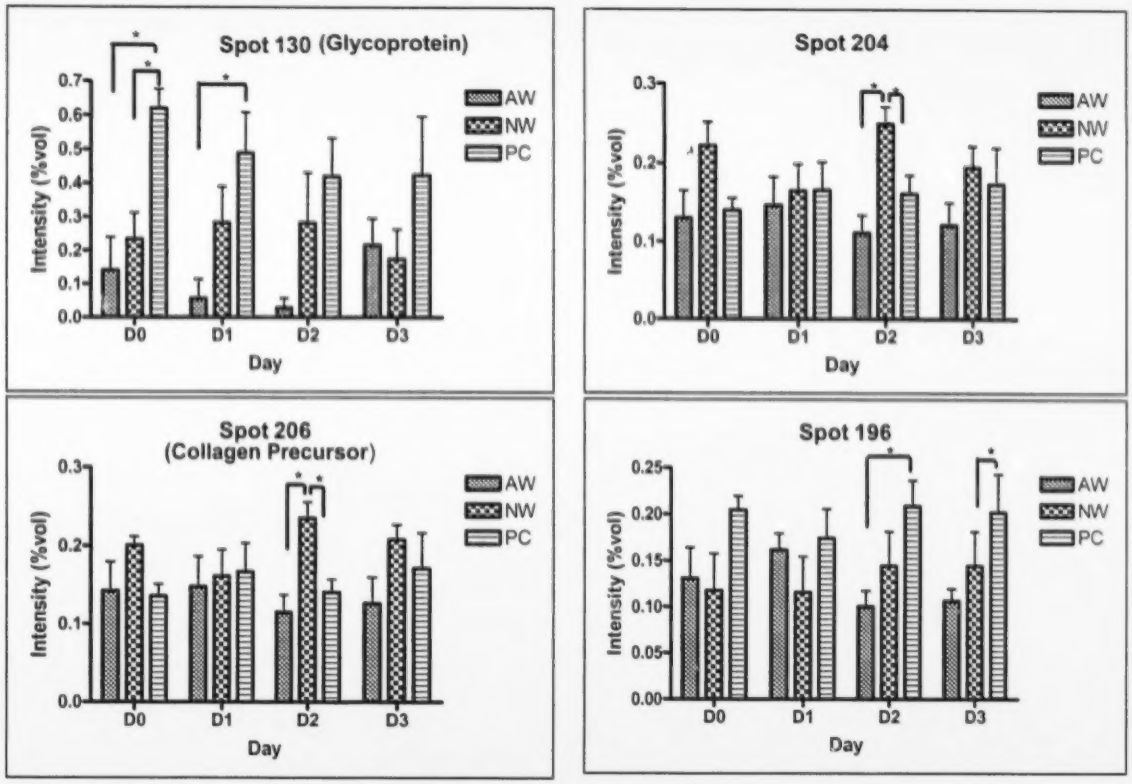


Table 5. Number of significant protein biomarkers in each group

NW Group	PC Group	AW Group
2	5	10

This is the final report of the three year project. The report clearly indicates that we were able to deliver all the milestones in due time and we are successful in initiating newer areas to understand stress dependent disease susceptibility. We have finished the planned animal experiments, sample collections and also identified protein biomarkers associated with BHV-1 infection as initially claimed in the proposal. We also archived nasal secretion sample for future studies. Identification of biomarkers and understanding of mechanism of stress dependent disease outcome needs further investigations. We initiated studies within this project to understand the effect of weaning stress in the absence of transportation which is another major cause of stress and disease susceptibility. We also initiated studies to understand the metabolite and elemental profile in serum samples. Results indicated that (a) we can predict stress condition and disease outcome by identifying biomarkers (b) a group of indicators for any particular

condition are more dependable than a single biomarker, (c) metabolites are more sensitive to predict disease outcome over other methodologies attempted.

The results for various conditions and outcomes are given below:

Stress: We identified serum amyloid A and apoCIII as biomarkers associated with abrupt weaning. Although we observed significant decrease in serum amyloid A abundance in abrupt weaned calves in comparison to pre-conditioned calves but the physiological significance of that can not be understood from the current study. Increased levels of apoCIII on Day 0 in AW-versus PC-calves suggested hypertriglyceridemia by delaying the catabolism of triglyceride-rich particles in weaned calves which can lead to increased infection susceptibility by altering the lipid transport and metabolism. This may be further supported by the observations that all significant metabolites (amino acids and LDLs) were higher immediately following weaning than in control calves.

Infection: We observed an increase in acute-phase serum proteins in all calves following BHV-1 infection which is consistent with a viral-induced inflammatory response. Increased apoAI in BHV-1 infected calves from both AW and PC calves may also imply promotion of cholesterol efflux from tissues to the liver for excretion as this is the major component of high density lipoprotein (HDL). We also observed elevated levels of haptoglobin in AW-calves than PC-calves following BHV-1 infection which is again consistent with enhanced inflammatory and acute phase response following viral induced infection. Haptoglobin is also known to bind with hemoglobin. Increased haptoglobin may reduce hemoglobin induced injury caused by whole blood for example in lung as a result of respiratory infection and can indicate enhanced hemoglobin catabolism in lung cells in order to protect lung injury . The mechanism however is not very clear. Acute phase proteins and various metabolites identified are also known to be directly related in controlling, regulating and interacting with various different pro-inflammatory cytokines and other proteins such as IL6, IL1, components of NFkB family and Insulin. Because a local increase in the concentrations of these pro-inflammatory cytokines precedes the development of acute signs and symptoms of illness, it has been implicated as a mediator in the pathway for symptom expression. We also observed increased levels of glucose, amino acids and LDLs in all calves following BHV-1 infection. Increased metabolite levels indicate change in metabolism which could lead to observed decrease in body weight following viral infection.

Disease Outcome: The proteomic comparison confirmed earlier observations that acute phase proteins can be significant indicators for calves which died and survived following secondary bacterial infection. No single protein biomarker was identified before (Day 0) or after infection (Day 4) when compared between calves which died and survived which could have predicted mortality before viral or bacterial infection.

Discriminant analysis of metabonomic data for calves which survived (n=8) and died (n=12) following secondary bacterial infection clearly identified a difference between these groups. When calves which survived and died were compared for significant metabonomic biomarkers, the profile suggested increased levels of lactate on Day 0 and glucose on day 4 in calves which survived secondary bacterial infection. Although detailed reason is not known but the significant higher abundance of lactate in survivor calves on Day 0 is consistent with significant lower abundance of glucose in these animals. It is known that under anaerobic condition glucose converts into lactate. Levels of LDL were higher on Day 4 for all animals but higher levels of glucose in surviving calves on Day 4 than Day 0 may be a biomarker which can differentiate the outcome of a secondary bacterial infection. Similarly increased Fe in Dead group of animals may explain the bacterial susceptibility of this group in contrast to Survived group which did not have any detectable level of Fe in serum.

Our next goal is to verify the results in a real life management situation to see if we can predict the stress level of calves and predict the outcome of natural infection. As a next step we would therefore like to get more funding to identify markers for each individual stressor which was beyond the scope of the current project as well as to understand the mechanisms of stress and disease outcome. The study successfully established various high-throughput methodologies to discriminate between stress and control and to correlate with disease outcome using serum samples, which could be a potential tool for producers to predict disease susceptibility and take measures to save huge financial losses incurred by BRD and other related diseases. However, our renewal attempt to further the knowledge was not successful consecutively last two years. It would be beneficial for the continuation of the current project and to obtain more relevant information to improve cattle health, handling and develop strategies for better protection to get this funding prioritized and renewed. We started the project a little later than expected because of restricted laboratory space due to construction at VIDO and a delay in recruiting graduate students, we have caught up with our proposed milestones and initiated new studies to identify the metabolite biomarkers for stress and disease.

Publications came out of this project:

1. Hodgson, P.D., Aich, P., Manuja, A., Hokamp, K., Roche, F.M., Brinkman, F.S.L., Potter, A., Babiuk, L.A., and Griebel, P.J. (2005): Effect of Stress on Viral-Bacterial Synergy in Bovine Respiratory Disease: A review of Potential Mechanisms, **Comparative and Functional Genomics 6**: 244-250
2. P. Aich, S. Jalal, C. Czuba, K. Herzog, G. Schatte, D. Olson, A. Ross, P.J. Griebel, A.A. Potter, L.A.Babiuk (2006) Serum Biomarkers for Stress, primary viral infection and Bovine Respiratory Disease Outcome **(in preparation)**
3. K. Herzog, H. Townsend, J. Stookey, P. Griebel, A.A. Potter, L.A. Babiuk and P. Aich (2006). Serum Biomarkers for Weaning Stress in Young Calves **(in preparation)**
4. S. Jalal, K. Herzog, P. Griebel, A. Potter, L. Babiuk and P. Aich (2005): A Proteomic Analysis of Serum Proteins following BHV-1 Iinfection in Cattle. **HUPO conference**, Aug 28-Sep 02, Munich, Germany.
5. K. Herzog, B. Sampathkumar, L.A. Babiuk, A. Potter, P. Griebel and P. Aich. Corticosteroid Induced Stress in Young Calves: A Proteomic Analysis,
    a. **Beyond Genome 2004**, June 21-24, 2004, San Francisco CA
    b. 11th Annual Life Sciences Student Research Day, May 13 and 14, 2004, U of Saskatchewan.
    c. Annual Graduate Student Research Poster Day, March 12, 2004, Dept. of Veterinary Microbiology, WCVM, U of Saskatchewan.

PERSONNEL

Graduate student, Katie Herzog started September 2003.

EQUIPMENT : See attached

PROJECT DEVELOPED MATERIALS: N/A

PROJECT PHOTOS: N/A

ACKNOWLEDGMENT: ADF is acknowledged for any publication, presentation and discussion of the current project.

EXPENSE STATEMENT: See Attached

ICAR Data Entry: Project proposal outline is entered on the ICAR. **ICAR-ID:** 33331355